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TITLE: Molecular Profiling of EPI-001: An Inhibitor of Androgen Receptor Signaling With a Disputed Mechanism of Action

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14. ABSTRACT
The goals of this project are to synthesize alkyne-functionalized EPI-001 analogues and to determine their protein-binding partners in prostate cancer cell lines. The anticipated outcome is the annotation of the activity-associated protein targets of EPI-001 that will be valuable in future drug development efforts. The progress achieved during this 1-year award include: (1) synthesizing a set of alkyne-tagged EPI-001 probes, (2) elucidating appropriate concentrations and incubation times for dosing probes to LNCaP cells to afford probe-protein adducts, (3) optimizing protocols for the isolation of probe-protein adducts over monomeric avidin resin and demonstrating protein-binding by SDS-PAGE, and (4) identifying the proteins captured by the EPI-001 analogues by LC-MS/MS. Ongoing studies are focused on performing quantitative proteomics with the prepared EPI-001 analogues to elucidate changes in protein levels following compound dosage. Additional control experiments and appropriate replicate experiments required for scientific publication are also being performed.

15. SUBJECT TERMS

Nothing listed

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INTRODUCTION:

The goals of this project are to synthesize alkyne-functionalized EPI-001 analogues and to determine their protein-binding partners in prostate cancer cell lines. The anticipated outcome is the annotation of the activity-associated protein targets of EPI-001 that will be valuable in future drug development efforts. The progress achieved during this 1-year award include: (1) synthesizing a set of alkyne-tagged EPI-001 probes, (2) elucidating appropriate concentrations and incubation times for dosing probes to LNCaP cells to afford probe-protein adducts, (3) optimizing protocols for the isolation of probe-protein adducts over monomeric avidin resin and demonstrating protein-binding by SDS-PAGE, and (4) identifying the proteins captured by the EPI-001 analogues by LC-MS/MS. Ongoing studies are focused on performing quantitative proteomics with the prepared EPI-001 analogues to elucidate changes in protein levels following compound dosage. Additional control experiments and appropriate replicate experiments required for scientific publication are also being performed.

KEYWORDS:

Prostate cancer, androgen receptor, EPI-001, protein pulldown, proteomics, target identification, organic synthesis, mass spectrometry, castration-resistant prostate cancer

ACCOMPLISHMENTS:

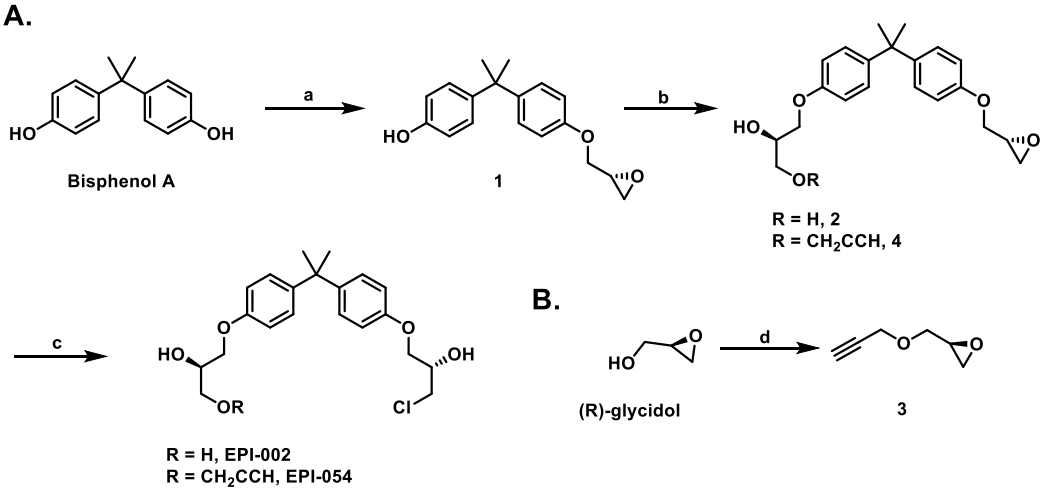
What were the major goals of the project?

Specific Aim 1: Synthesize and biochemically characterize EPI-family compounds
Subtask 1: Synthesize compounds **1**-**6**.
Subtask 2: Characterize the AR inhibitory activity of **1**-**6**.
Specific Aim 2: Perform activity-associated protein profiling with EPI-family compounds
Subtask 1: Perform cell-based studies with probes 1-6. Prepare samples for proteomics analysis. Submit samples for analysis.
Subtask 2: Annotate proteomics data. Generate list of activity-associated protein targets.
Subtask 3: Perform validation studies with activity-associated protein targets.

What was accomplished under these goals?

Specific Aim 1: Synthesize and biochemically characterize EPI-family compounds
Subtask 1: Synthesize compounds **1**-**6**.

Accomplishments: Three of the proposed six compounds were synthesized and fully characterized by NMR spectroscopy and mass spectrometry. EPI-002 and EPI-054 were synthesized in three linear steps starting from commercial materials. The synthesis of EPI-002 begins by coupling Bisphenol A with (S)-glycidol employing the Mitsunobu reaction to give mono-alkylated **1** in 49% yield (**Scheme 1**). The phenol of intermediate **1** opens the epoxide of (R)-glycidol in the presence of K_2CO_3 at 50 °C to give the diol **2** in 49% yield. The epoxide of **2** is opened with $CeCl_3$ in refluxing MeCN to give EPI-002 (74% yield, overall yield over three steps is 18%). EPI-054 is synthesized using the same strategy, but (R)-glycidol is first coupled to propargyl bromide using a Williamson ether synthesis to give **3** (60% yield, **Scheme 1B**), which is then reacted with intermediate **1** to form the propargyl ether-epoxide **4** in 28% yield. Opening of the epoxide ring with $CeCl_3$ produces EPI-054 in 79% yield (11% overall yield over three steps). EPI-001 was synthesized in the same manner as EPI-002, but racemic glycidol was used to obtain a mixture of four stereoisomers.



Scheme 1. Synthesis of EPI-002 and EPI-054.[a]
[a]**Reagents and Conditions:** (a) (S)-glycidol, Ph_3P, DIAD, THF, 49%; (b) NaH, DMF, (R)-glycidol or **3**, 80 °C, 49% for **2**, 28% for **4**; (c) $CeCl_3$, MeCN, reflux, 74% for EPI-002, 79% for EPI-054. **B.** Synthesis of propargyl (R)-glycidol precursor **3**. (**d**) NaH, propargyl bromide, TBAI, THF, 60% yield.

Specific Aim 1: Synthesize and biochemically characterize EPI-family compounds

Subtask 2: Characterize the AR inhibitory activity of **1**-**6**.

Accomplishments: To determine the effect of the propargyl group on EPI-054 compared to EPI-002, both compounds were tested for their cytotoxic properties against LnCaP cells. In a 48 h cytotoxicity assay with LnCaP cells (**Table 1**), EPI-002 has a high micromolar IC_{50} value of 108 ± 5 μM. EPI-054 also has a high micromolar IC_{50} value of 61 ± 6 μM, approximately a 2-fold difference compared to EPI-002.

Table 1. Cytotoxicity of EPI-002 and EPI-054 in LnCaP cells. Cells were dosed for 48 h and cell viability was indirectly determined by metabolic activity using Alamar Blue. IC_{50} values are Mean ± S.D. (n ≥ 3).

Compound	IC_{50} ± S.D. (μM)
EPI-002	108 ± 5 μM
EPI-054	61 ± 6 μM

The alkyne derivative EPI-054 was further tested for its effects on AR mRNA expression levels compared to that of EPI-001 (**Figure 1**). Both compounds were dosed to LnCaP cells at 50 μM for 16 h with or without 1 nM of dihydrotestosterone (DHT). The AR mRNA expression levels were determined using qRT-PCR. Dosing DHT to LnCaP cells has a modest effect on AR mRNA expression levels compared to the non-treated control. EPI-001 and EPI-054 have similar inhibition of AR mRNA expression levels in the presence or absence of DHT, and suggests that EPI-001 and EPI-054 inhibit AR mRNA expression indirectly of AR. Approximately a 50% reduction in AR mRNA levels occurs when EPI-001 and EPI-054 are dosed at 50 μM compared to the non-treated control. These data suggest that EPI-001 and EPI-054 have the same effect on AR expression levels and behave similarly in LnCaP cells. Thus, EPI-054 serves as a probe mimic for EPI-002 for protein identification studies.

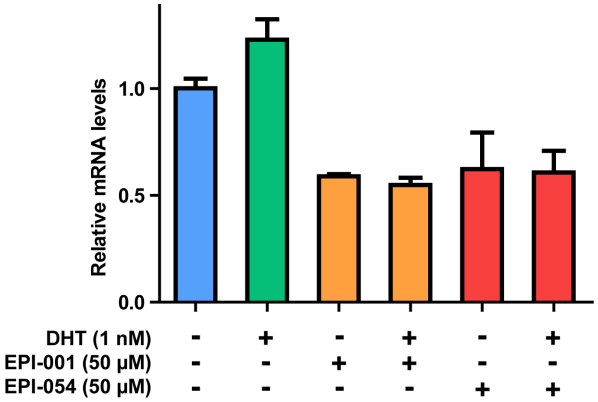


Figure 1. Relative mRNA levels of AR were determined in LnCaP cells. EPI-001 and EPI-054 were dosed to LnCaP cells at 50 μM for 16 hours with or without dihydrotestosterone (1 nM). Expression levels were normalized to AR mRNA level of non-treated LnCaP cells and GAPDH mRNA control. Data shown is the mean relative mRNA level ± S.D. (n = 3).

Specific Aim 2: Perform activity-associated protein profiling with EPI-family compounds

Subtask 1: Perform cell-based studies with probes 1-6. Prepare samples for proteomics analysis. Submit samples for analysis.

Accomplishments: Cell based studies with EPI-054 and EPI-002 (utilized in competition assays as a control) was performed to elucidate appropriate concentrations and incubation times for dosing molecules to LnCaP cells to achieve protein labeling. Once these conditions were optimized, those compounds were used in cells and protein pulldown was performed. Pulldown conditions were optimized and protein isolation was confirmed. Finally, one sample set was repeated and submitted for proteomics analysis in-house.

To demonstrate that EPI-054 labels multiple proteins within the LnCaP cell proteome an in-gel fluorescent labeling assay was used. LnCaP cells were treated with 50 μM of EPI-054 for 36, 24, and 16 hours (**Figure 2**). Previous reports suggest that 24 h exposure of EPI-054 is optimal. After dosing the cells for the time indicated the cells were lysed via three freeze thaw cycles and the lysate was cleared by centrifugation. Lysate protein concentrations were normalized to the lowest value and TAMRA-N_3 was attached to the EPI-054-protein adducts with the copper catalyzed [3+2] Huisgen reaction (click chemistry). The proteins were separated with a denaturing PAGE gel and labeled proteins were visualized. The most significant labeling intensity was seen at the 24 h time point. After visualization of the labeled proteins, Oriole® total protein stain was use to show equal total protein concentration loading. Notably, at the 36 h time point the total protein is slightly reduced compared to other time points potentially because of premature cell death due to the long incubation time with EPI-054. The IC_{50} value of EPI-054 is 61 ± 6 μM, so presumably significant cell death would occur at 50 μM for 36 h. Therefore, 24 h incubation was determined to be optimal for labeling experiments.

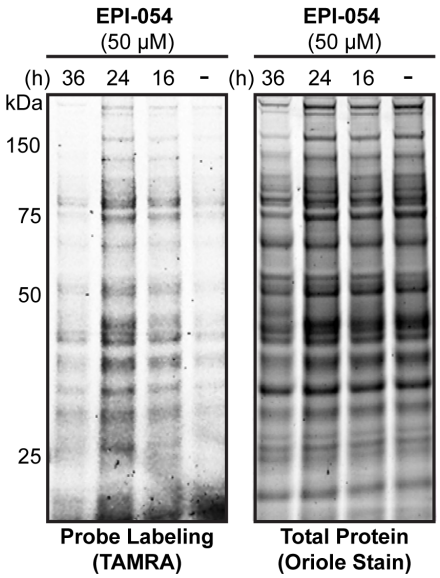


Figure 2. In-gel fluorescence labeling of EPI-054 in LnCaP cells. Cells were dosed with 50 μM of EPI-054 or a DMSO control for 36, 24, and 16 h. The cells were lysed and TAMRA-N_3 was attached to the EPI-054-protein adducts for visualization. After visualization of labeled protein, the gel was stained with Oriole® total protein state to demonstrate equal protein loading.

After determining the optimal time for dosing, LnCaP cells were dosed with a DMSO control or EPI-054 at 100, 50, and 25 μM for 24 h (**Figure 3**). Cells were lysed and protein adducts were conjugated to TAMRA-N_3 in the same manner as the previous labeling experiment (*vide supra*). The fluorescent signal for labeled proteins was strongest at 100 μM, but significant cell death occurred (50% cell viability detected with trypan blue) over the 24 h period. At 50 and 25 μM the same bands can still be visualized compared to the 100 μM dose, but less cytotoxicity was observed (80% cell viability detected with trypan blue). Because of the differences in cell viability, a 50 μM dose was chosen for target identification studies.

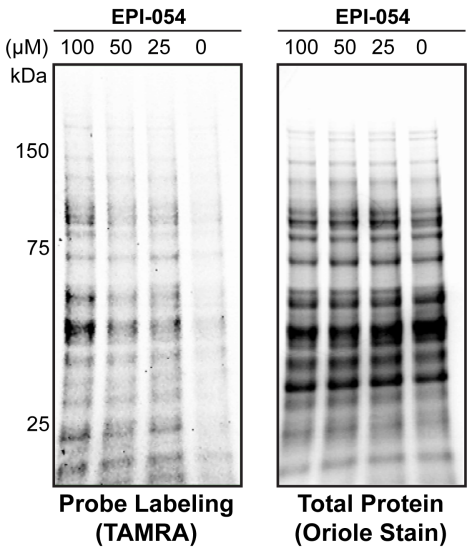


Figure 3. EPI054 was dosed to LnCaP cells at 100, 50, and 25 μM or a DMSO control for 24 hours. The cells were lysed and TAMRA-N_3 was attached to the probe-protein adducts via click chemistry. After labeling visualization the gel was stained with Oriole total protein stain to demonstrate equal lane loading.

Specific Aim 2: Perform activity-associated protein profiling with EPI-family compounds
Subtask 2: Annotate proteomics data. Generate list of activity-associated protein targets.

Accomplishments: We have treated LnCaP cells with EPI-054 to identify potential protein targets within the cell proteome. EPI-054 was treated to LnCaP cells at 50 μM for 24 h or first treated with 50 μM of EPI-002 4 h prior to addition EPI-054 at 50 μM, and then incubated for an additional 20 h as a competition experiment. After treatment, the cells were lysed and biotin-N_3 was attached to protein-probe adducts via click chemistry. The probe-protein adducts were enriched using monomeric avidin and the enriched samples were analyze using LC-MS/MS. We have generated a preliminary list of protein targets of EPI-054. Hundreds of potential protein targets have been identified, and we are currently running replicates and controls that are needed for validation of our results. Additionally, we are currently performing quantitative proteomics analysis to determine how our probes affect global protein levels in prostate cancer cells. These studies are ongoing.

Specific Aim 2: Perform activity-associated protein profiling with EPI-family compounds
Subtask 3: Perform validation studies with activity-associated protein targets.

Accomplishments: We have performed a Western blot analysis for androgen receptor after pulldown using EPI-054 to corroborate identification using a proteomics-based approach. After treatment of cells with EPI-054 and EPI-002 in the same manner as described above, the cells were lysed, biotin-N_3 was attached to probe-protein adducts via click chemistry, and protein-probe adducts were enriched. EPI-054 pulls down androgen receptor evidenced by Western blot analysis after dosing EPI-054 (**Figure 4**), but when EPI-002 is dosed prior to EPI-054 the interaction is partially ablated. These data suggests that EPI-054 and EPI-002 directly interact with androgen receptor, the previously reported primary target. Studies are ongoing to verify additional protein targets using Western blot analysis and other experimental methods.

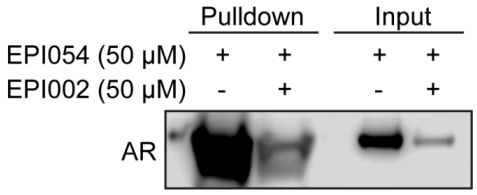


Figure 4. Androgen receptor was pulled down after treating LnCaP cells with EPI-054 and identified using Western blot analysis. In a competition experiment, EPI-002 ablated the interaction between the androgen receptor and EPI-054.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report (this is the final report)

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Previously, the N-terminal domain of androgen receptor has been described as the primary target of EPI derivatives for inhibition of prostate cancer proliferation. It is clear from previous studies in our laboratories and the data presented here that EPI-054, EPI-002, and

other derivatives covalently modify multiple proteins within LnCaP cells. Identifying off-target proteins that interact with EPI derivatives is important to understanding the biological mechanism of action of these compounds and how to improve specificity for targeting prostate cancer cell proliferation. Although still ongoing, these studies will likely clarify the mechanism of action by which EPI-001, EPI-002, and related analogues inhibit prostate cancer cell growth. Such results will be informative in the design and development of new therapies to treat prostate cancer, as well as in the optimization of EPI-family of compounds for clinical applications.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Elucidating the concentrations required for each probe and the incubation time that each probe needed on cells to elicit appropriate levels of protein labeling without cellular toxicity required a significant amount of optimization. This problem is resolved, but resulted in a delay in the overall progress of the project. Although this award has ended, we anticipate delivering on the final goal of elucidating the activity-associated protein targets of EPI-001 analogues.

Change that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

PRODUCTS:

Journal publications:

Wells SM, Widen JC, Harki DA and Brummond KM. Alkyne ligation handles: Propargylation of hydroxyl, sulfhydryl, amino, and carboxyl groups via the Nicholas reaction. *Org. Lett.* **2016**, *18,* 4566-4569.

Conference abstracts:

Nothing to report

Presentations:

Chemical Biology Interface Training Grant Symposium; Minneapolis, MN (2015). Poster Presentation: *Development of a Natural Product-inspired Covalent Probe Towards the Transcription Factor p65/RelA of the NF-κB Pathway*. John C. Widen (presenter), Aaron M. Kempema, and Daniel A. Harki.

American Chemical Society, Division of Organic Chemistry, Graduate Research Symposium, St. Edward's University, Austin, Texas (2015). Poster Presentation: *Synthesis of Natural Product Based RelA/p65 Probes for Targeted Inhibition of the NF-κB Pathway*. John C. Widen (presenter), Aaron M. Kempema, and Daniel A. Harki.

54th Annual MIKI Medicinal Chemistry Meeting; Iowa City, IA (2016). Oral Presentation: *Development of Cysteine Reactive Probes Towards p65/RelA of the NF-κB Pathway.* John C. Widen (presenter), Aaron M. Kempema, Peter W. Villalta, and Daniel A. Harki.

Bioorganic Chemistry Gordon Research Conference; Andover, NH (2016). Poster Presentation: *Development of Cysteine Reactive Probes Towards p65/RelA of the NF-κB Pathway.* John C. Widen (presenter), Aaron M. Kempema, Peter W. Villalta, and Daniel A. Harki.

Website or other Internet Site(s):

Nothing to report

Technologies or techniques:

Nothing to report

Inventions/patent applications, and/or licenses:

Nothing to report

Other products:

Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANICATIONS:

What individuals have worked on the project? (1 month or greater)

Name	Project Role	Person Months	Contribution
Daniel Harki	PI	1.2	Oversaw all aspects of the project, including experimental design, data analysis, and problem solving. Responsible for grant reporting.
John Widen	Graduate Student	4.1	Synthesized compounds, performed cellular protein labeling studies, performed proteomics.
Mexia Che	Postdoc	1.4	Performed biochemical studies to determine the androgen receptor mRNA following treatment with probes.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Daniel Harki (PI), is now co-I on a new NIH grant: R01-GM118000 (began 1/17/2016)

What other organizations were involved as partners?

Nothing to report

SPECIAL REPORTING REQUIREMENTS:

Nothing to report

APPENDICES:

Nothing to report